Facile Method for Monitoring Inhibition of Anaerobic Spore Outgrowth

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A device is presented for the laboratory monitoring of spore outgrowth under controlled temperature and anaerobic conditions. Alterations in pH, redox potential, headspace composition, and optical density are followed as the activated spores grow out into vegetative cells. An interlock system allows the addition of test solutions or the removal of medium under anaerobic conditions. The device may also be used for rapid (<4 h) chemical inhibition studies or adapted for temperature injury studies of aerobic or anaerobic cells. Data on outgrowth of *Clostridium sporogenes* and inhibition by nitrite solutions are presented.

The biochemistry of anaerobic organisms has not been studied to the same extent as that of aerobic organisms because of the difficulty in maintaining anaerobiosis during growth and subsequent analyses. Several species of the anaerobic sporeforming clostridia, including *Clostridium tetani* (tetanus), *C. welchii* (gangrene), *C. perfringens* (food intoxications), and *C. botulinum* (botulism) are important from a medical standpoint because of the pathogenic toxins formed (1).

For our studies on the mechanism of inhibition by nitrite on the outgrowth of sporeforming clostridia, including *C. sporogenes*, we developed a simple laboratory anaerobic growth chamber which permits monitoring of growth parameters (pH, temperature, redox potential, and absorbance) and allows the addition of test solutions or the removal of samples under anaerobic conditions. Although designed for spore outgrowth studies as they pertain to foods, this apparatus is equally useful for vegetative cell growth experiments or for cell injury studies.

MATERIALS AND METHODS

The device consisted of a water-jacketed glass cylinder (100-ml capacity) with an open top and entrance and exit ports at the bottom (Fig. 1). The medium within the vessel was agitated by a magnetic stirrer and was analyzed by being pumped through a flow cuvette in a spectrophotometer (Beckman DB-G) by means of a variable-rate peristaltic pump (Gilson Minipuls II) at 4 to 8 ml/min. The reaction cylinder was closed with a rubber stopper containing a combination pH electrode, a combination platinum electrode for measuring redox potential, a mercury thermometer, and a gas exit port. In operation, the vessel was filled to the brim with the appropriate medium before the stopper was inserted. Excess medium or gases were removed by the gas port, and the medium was allowed to equilibrate to the desired temperature while background readings were made. Spore mixtures, activated when required, and test medium additives, when used, were added through the valve interlocks (Fig. 1). Both four-way valves were turned clockwise one stop at the same time to divert the flow to the parallel tubing, and the medium in the diverted tubing was removed or replaced or both. The diverted tubing should be of known internal volume to provide a sample for subsequent microbiological or chemical analysis. The sample can be flushed through with the syringe containing nitrogen gas to maintain anaerobiosis. The valves were then turned one stop clockwise to reinsert the tubing in the flow. Alternatively, for continuous medium removal and replacement, only one valve need be turned.

The device was cleaned after each experiment by flushing with water, detergent solution, water, the appropriate sanitizer solutions (hypochlorite or quaternary ammonium compounds), and water. Background runs (no added spores) were conducted to monitor sanitation. The device can be disassembled, the glass vessel can be autoclaved, and the polyethylene or Teflon tubing can be replaced for complete sterility. In these experiments to show the effect of nitrite or pH or both on spore outgrowth, the incubation medium was a modification of that described by Huhtanen (4) and contained the following (grams per liter): yeast extract, 5.0; tryptone, 5.0; nutrient broth, 2.65; dipotassium phosphate, 1.2; glucose, 2.0; and sodium thioglycolate, 0.5. The pH was adjusted to the desired value (6.5 or 5.5) with hydrochloric acid.

The free nitrite concentration in the medium was determined by a modified Griess-type reaction. To 0.5 ml of sample were added 4.0 ml of distilled water and 0.5 ml of Griess reagent (1.0 g of sulfanilic acid and 0.316 g of 8-amino-2-naphthalenesulfonic acid sodium salt in 600 ml of 15% acetic acid). Samples were mixed thoroughly, and absorbance at 520 nm was determined after 15 min of incubation at room temperature. With nitrite standard solutions, the absorbances were linear for up to 1 mM concentrations. In media in which nitrite was added, the concentration was 150 ppm (150

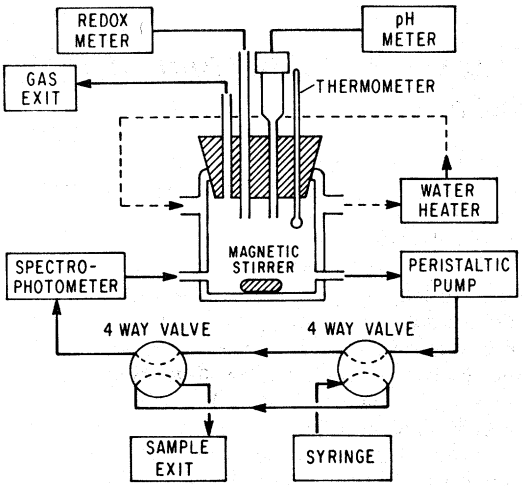

FIG. 1. *Diagram of the growth vessel and associated control and monitoring devices.*

μg/ml), and was either autoclaved in the medium (heated nitrite medium) or added separately (sterilized) to the autoclaved medium (unheated nitrite medium).

RESULTS AND DISCUSSION

C. sporogenes B1219 (ATCC 7955) was used as the test organism because it is biochemically similar to the proteolytic strains of *C. botulinum*, but is nontoxigenic. Spores were prepared by the method of Santo Goldini et al. (7). Figure 2 shows the growth of this organism from heat-activated spores (80°C for 10 min; direct microscopic count in test suspensions, 4.7×10^4 spores per ml) in the modified media at pH 6.5 and 5.5 with or without 150 ppm of sodium nitrite. Perigo and Roberts (6) noted that when a culture medium containing nitrite is autoclaved, a substance (not nitrite) that actively inhibits growth of *C. botulinum* is formed, presumably by a thermal reaction of nitrite with unknown components of the medium. The effect of heated nitrite medium and unheated nitrite medium at pH 5.5 is also graphed. The nitrite for addition was sterilized separately by autoclave.

In these experiments, growth was measured by increases in absorbance readings at 600 nm. Direct microscopic examination of samples during h 1 of incubation indicated that initial increases in absorbances were produced by increased light scattering by swollen spores and by some spore outgrowth. The combined effect of initial pH and added nitrite in the medium was most evident at 3 h after inoculation. Growth was more rapid at the higher pH, but the higher apparent initial growth at the lower pH was produced by an increased turbidity effect, basis unknown. When a medium with no added nitrite and an initial pH of 6.5 was incubated for 3 h at 31°C, the absorbance (600 nm) increased by 0.608 U, the pH decreased by 0.86 U, and the redox potential decreased by 125 mV (uncorrected). Corrections for the pH effect on redox potentials may be made when necessary (2). The addition of separately sterilized nitrite to the medium (pH 6.5, 0.001 M phosphate) initially had little effect, but as the pH of the medium decreased to below 5.7 after 2.5 h as a result of the outgrowth of the spores and acid production by the clostridia, the content of the inhibitory nonionic nitrous acid ($pK_a = 3.36$) increased and slowed growth (6). In the presence of nitrite (Fig. 2), apparent growth in the medium at pH 6.5 was 57% of that in the absence of nitrite. Comparative figures apply to absorbances at 600 nm after 3 h of incubation. In the medium with the lower initial pH (5.5), growth was 27% of that at 6.5, with both samples free of added nitrite. Growth in the heated nitrite medium was 82% of its control, and growth in the unheated nitrite medium was 40% of its control. After 3 h (initial pH 5.5), the apparent nitrite concentration of the heated nitrite medium had decreased to 92 ppm, and the absorbance had increased by 0.107 U. In similar experiments, there was a significant

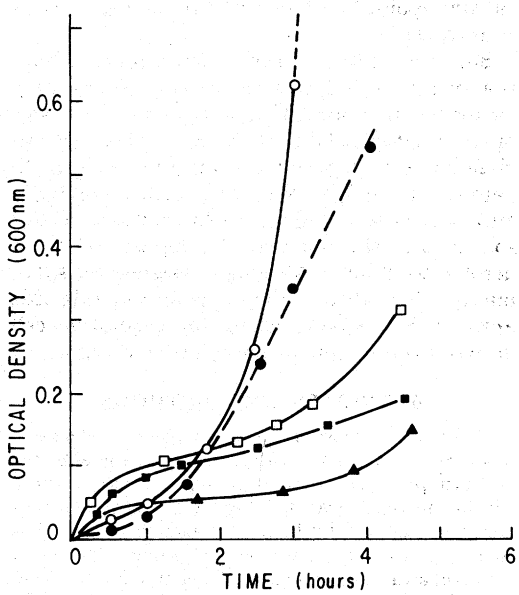

FIG. 2. *Outgrowth (absorbance at 600 nm) of C. sporogenes spores at 31°C under anaerobic conditions as a function of pH and nitrite level. Symbols: ○, pH 6.5, no nitrite; ●, pH 6.5, 150 ppm of nitrite; □, pH 5.5, no nitrite; ■, pH 5.5, 150 ppm of nitrite before autoclaving; ▲, pH 5.5, 150 ppm of nitrite after autoclaving.*

negative relationship ($r = -0.61$; $P = 0.05$) between growth of the cells and the nitrite concentration. The biochemical mechanisms for these findings are being investigated (1).

The temperature, spore concentration, and medium used in these experiments were chosen to give the most rapid growth responses within a model system. *C. sporogenes* and *C. botulinum* grow optimally in the temperature range of 25 to 35°C. The same medium has been employed at pH 6.8 for long-term inhibition studies with *C. botulinum* cells (5). The present experiments, however, used 5.5 as a more representative pH of cured meat products (range, 5.3 to 6.0) with nitrite used as an additive. Under these conditions, an indication of possible inhibition by an agent can be obtained within 4 h. With use of a more restrictive medium (3), detectable growth occurred only after 6 to 10 h; the yield of cells was approximately 15% of that from the enriched medium.

This apparatus also may be used in other studies in which the effect of an alteration of a particular parameter is to be examined. With continuous monitoring of pH, redox potential, temperature, and growth, interactive effects may be noted. Cell injury or death produced by increased incubation temperatures can often be followed spectrophotometrically by increases in ultraviolet-absorbing material released into the medium (J. L. Smith, R. C. Benedict, and S. A. Palumbo, manuscript in preparation). With this method of monitoring during temperature exposure, the rate of injury may be more closely followed. The method further permits differentiation of such effects under aerobic and anaerobic conditions.

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